

Research articles

Accurate Assessment of Alpha-gal Syndrome using Cetuximab and Bovine Thyroglobulin-specific IgE

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Abbreviations: α -Gal, galactose- α -1,3-galactose; BTG, bovine thyroglobulin; ROC, receiver operator characteristic; sIgE, specific IgE; SPT, skin prick test.

Key words: α -Gal; Alpha-gal syndrome; Bovine thyroglobulin-specific IgE; Cetuximab-specific IgE; Component-resolved diagnosis.

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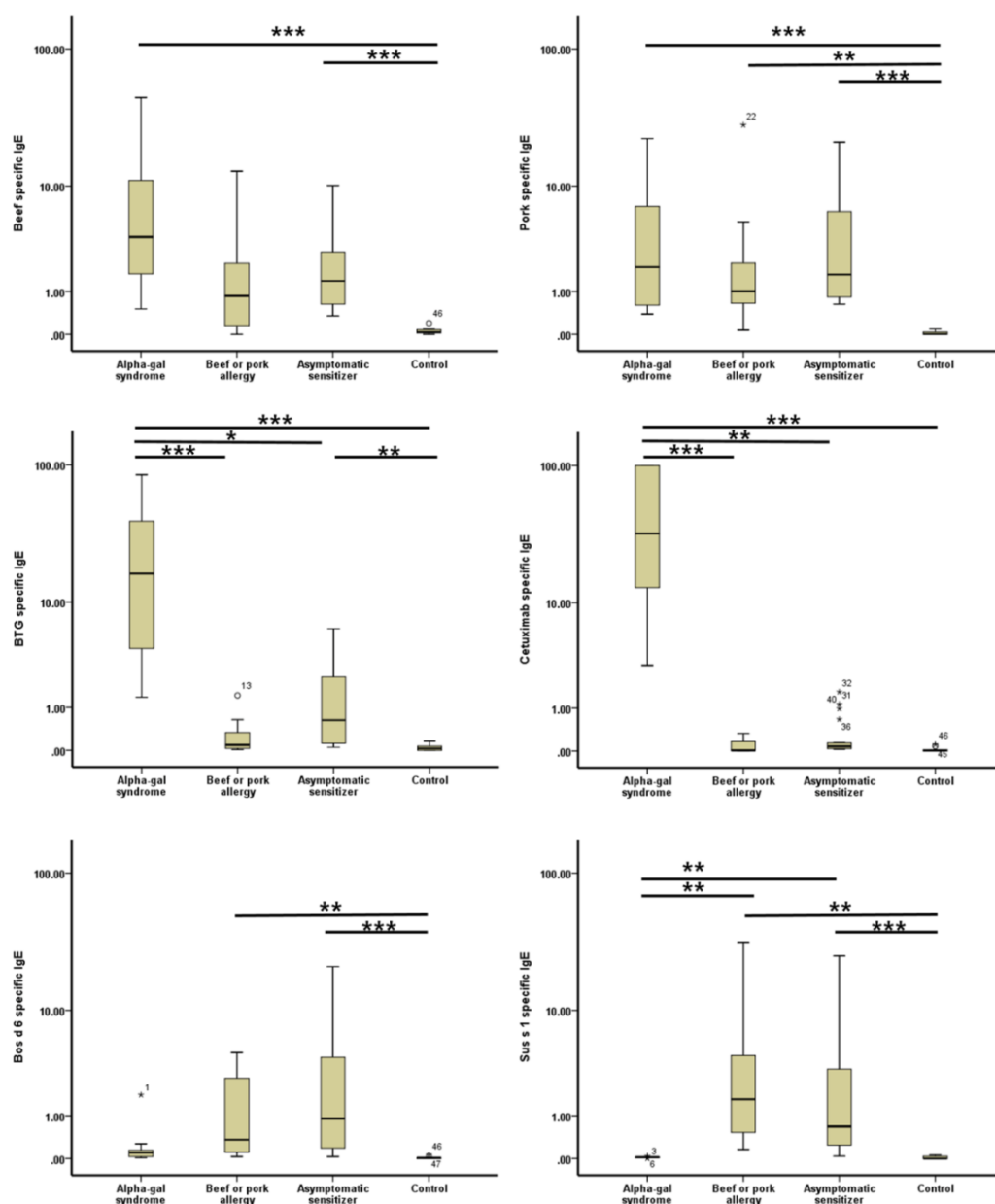
Abstract

Scope: IgE against galactose- α -1,3-galactose (α -Gal) cause alpha-gal syndrome. Bovine thyroglobulin (BTG) and cetuximab share this epitope. We aimed to determine the utility of specific IgE (sIgE) against cetuximab as compared to BTG for diagnosing alpha-gal syndrome.

Methods and results: Twelve patients with alpha-gal syndrome, 11 patients with immediate beef or pork allergy, 18 asymptomatic individuals with meat sensitivity, and 10 non-atopic subjects were enrolled. We checked the levels of sIgE against BTG and cetuximab using the streptavidin CAP assay. Additionally, IgE reactivity to BTG and cetuximab was assessed by immunoblotting. All alpha-gal syndrome patients had a high concentration of sIgE against BTG, and cetuximab. In contrast to alpha-gal syndrome, patients with immediate allergic reactions to meat consumption and those with asymptomatic sensitization had significantly lower concentration of BTG and cetuximab sIgE, and a high prevalence of sIgE against bovine or porcine serum albumin. Although the concentration of sIgE against alpha-gal was lower in individuals with asymptomatic sensitization, IgE immunoblotting showed the presence of sIgE against α -Gal in this group.

Conclusion: Differentiation of alpha-gal syndrome from patients with immediate allergy to meat consumption or asymptomatic sensitization requires quantification of cetuximab- or BTG-induced sIgE via detection of IgE for α -gal.

Figure shows the results for the sIgE assays for beef, bovine serum albumin, pork, porcine serum albumin, BTG, and cetuximab for each group. Patients with alpha-gal syndrome had significantly higher sIgE concentrations to beef, pork, BTG, and cetuximab than controls. However, these patients showed low sIgE concentrations in response to bovine and porcine serum albumin, similar to those observed in healthy controls. The sIgE against cetuximab and BTG could effectively discriminate between alpha-gal syndrome and the other three groups.



1 Introduction

Food allergies affect 2–10% of the population [1,2], and their prevalence has increased in the last several decades worldwide [3]. Food allergies have become a public health concern that can affect the overall quality of life and lead to life-threatening reactions such as anaphylaxis [4]. An immediate response after ingesting food is a classic symptom of food allergy resulting from immunoglobulin E (IgE)-mediated hypersensitivity. However, over the last decade, investigators have reported a new type of food allergy to red meats, which presents as a severe hypersensitivity reaction with a delay of several hours in adult subjects [5,6].

Alpha-gal syndrome was the first food allergy showing IgE-mediated delayed-onset anaphylaxis, angioedema, or urticaria [6]. A specific IgE (sIgE) to galactose-alpha-1,3-galactose (α -gal) was identified as the cause of alpha-gal syndrome [6]. α -Gal is found in all mammals apart from Old World monkeys, apes, and humans [7-9]. It is speculated that patients develop sensitization to α -gal through tick bites [10-12], parasites [13], or exposure to cats [14] or cetuximab, which is a chimeric monoclonal antibody approved for use in squamous cell carcinoma of the head and neck and colorectal cancer [15]. α -Gal is known to exist in bovine thyroglobulin (BTG) [16] and the murine heavy chain portion of cetuximab [17]. In addition, cetuximab and BTG have been shown to be recognized by IgE from patients with alpha-gal syndrome [15,16].

Thus far, skin prick tests (SPTs) and sIgE testing for commercial crude meat extracts such as beef, pork, or lamb in alpha-gal syndrome patients generally yield poor or negative results [18]. Therefore, several other methods have been suggested recently for diagnosis of alpha-gal syndrome [18-21]. To date, most studies have focused on the diagnosis of alpha-gal

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syndrome, and no studies have addressed how to distinguish alpha-gal syndrome from individuals with asymptomatic sensitization or patients with immediate allergic responses to meats. We studied the clinical values of α -gal sIgE detection using BTG and cetuximab for distinguishing alpha-gal syndrome from patients with immediate allergic responses or asymptomatic meat sensitivity.

2 Materials and methods

2.1 Study subjects

In total, 51 patients who had visited the Allergy and Asthma Center of Severance Hospital (Seoul, Korea) between September 2014 and December 2016 were enrolled in this retrospective study. Among them, 39 patients had at least one allergic disease, and 31% of the patients had more than two allergic diseases (Table 1). This study was approved by the Institutional Review Board of Yonsei University College of Medicine (IRB No. 4-2015-0931) and all patients provided written informed consent.

We planned to analyze samples from four distinct groups for comparison with commercial products, and to estimate the inter-rater reliability of each method in confirming the diagnosis of alpha-gal syndrome. In group 1, serum samples were collected from 12 patients who suffered from recurrent severe delayed anaphylactic reactions with urticaria after ingestion of beef or pork. Group 2 consisted of 11 patients with immediate hypersensitivity reactions after ingestion of red meat, representing classical beef or pork allergy. Groups 3 and 4 were control serum samples for comparison with group 1. Group 3 consisted of 18 subjects who were positive for sIgEs against beef and/or pork without any allergic symptoms related to the ingestion of red meats, and therefore represented subjects with asymptomatic sensitization to red meats. Group 4 consisted of 10 non-atopic patients visiting the Allergy and Asthma Center for consultation who were referred from another department for suspected allergic disease. In these patients, the examination ultimately revealed no allergic disease with no symptoms of food allergy; therefore, this group was classified as the healthy control.

2.2 Review of medical records

Clinical data collection involved a review of medical records. The physicians assessed each record for a history of hypersensitivity reactions after ingestion of red meats. We collected the patients' clinical data using three main methods: (1) review of medical records only, (2) interview at the Allergy Asthma Center, and (3) telephone calls. The timing of the occurrence of the food allergy was recorded. The following additional information was

collected from the medical record: participant age and sex, presence or absence of other allergic disease, family history of allergic disease, total IgE levels, sIgE results for commercial extracts of red meats (beef and pork), and presence of other sensitization. We investigated the presence of other sensitization, i.e., other than sensitization to beef and pork, using SPTs, and the ImmunoCAP assay (Thermo Fisher Scientific, Phadia AB, Uppsala, Sweden). Total and specific IgE antibodies against red meats (beef and pork) were measured using the commercially available ImmunoCAP system. The assays were performed with the ImmunoCAP 250 instrument, and the results are expressed as international units per liter. For specific assays, allergen sIgE values ≥ 0.35 kU_A/L were regarded as positive [22].

2.3 Mammalian meat sIgE assays

The measurements were performed by a technician who was blind to the grouping. The commercial streptavidin CAP kit (Thermo Fisher Scientific, Phadia AB, Uppsala, Sweden) was used for detection of α -gal sIgEs [23]. To detect sIgEs against α -gal, BTG (Sigma-Aldrich, St. Louis, MO, USA) and cetuximab (Merck KGaA, Darmstadt, Germany) were biotinylated using EZ-Link[®] Sulfo-NHS-LC-Biotin (Thermo Fisher Scientific, Waltham, MA, USA). In brief, 1 mL of antigen (2 mg/mL for cetuximab or BTG) dissolved in phosphate-buffered saline (PBS) was added to 27 μ L of 10 mM biotin reagent. The reaction mixture was kept on ice for 2 h, and unreacted NHS-LC-biotin was removed by extensive dialysis against PBS. The biotinylated antigens were aliquoted and stored at -20 °C until use. Subsequently, sIgEs against these antigens were measured by ImmunoCAP. In addition, levels of sIgE against bovine serum albumin (Bos d 6) and porcine serum albumin (Sus s 1) were measured using the commercially available ImmunoCAP system.

2.4 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Antigens were dissolved in reducing sample buffer (12.5 mM Tris-HCl; pH 6.8, 5% glycerol, 0.4% SDS, 1% mercaptoethanol, and 0.02% bromophenol blue) and boiled at 100°C for 5 min. Allergens were separated by electrophoresis on 13.5% polyacrylamide gels (Hoeffer, San Francisco, CA, USA) at 50 V for 30 min, followed by 180 V for 2 h. The protein band of cetuximab was excised after staining with Coomassie brilliant blue R250 and subjected to LC-coupled ESI-MS/MS analysis for confirmation of protein identity at Yonsei Proteome Research Center (Seoul, Korea).

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2.5 IgE immunoblotting and inhibition of immunoblotting

Allergens were separated on 13.5% polyacrylamide gels contacting SDS under reducing conditions. The separated proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (GE Waters and Process Technologies, Trevose, Pa., USA). After blocking with Protein-Free T20 (TBS) blocking buffer (Pierce), sera were incubated overnight at 4°C. Membranes were incubated with a 1:1,000 dilution of alkaline phosphatase-conjugated goat anti-human IgE (Sigma) for 60 min. After washing, the colorimetric reaction was developed with the BCIP/NBT system (Promega, Madison, WI, USA). The buffer and secondary antibody (i.e., no primary antibody) served as the negative control. Anti- α -Gal monoclonal antibody (IgM type; Enzo Life Sciences, Inc., Farmingdale, NY, USA) served as the positive control, and HRP-conjugated goat anti-mouse IgM antibody (Kirkegaard & Perry Laboratories, Inc., KPL, Gaithersburg, MD, USA) was used.

To inhibit immunoblotting, the pooled sera from alpha-gal syndrome patients and α -gal sIgE-positive asymptomatic patients were preincubated with the appropriate concentration of cetuximab or BTG at room temperature for 2 h. This process bound up any reactive antibodies. Membranes were incubated with a 1:1,000 dilution of alkaline phosphatase-conjugated goat anti-human IgE (Sigma) for 60 min. After washing, the colorimetric reaction was developed with the BCIP/NBT system (Promega, Madison, WI, USA).

2.6 Statistical analysis

The baseline characteristics of the study subjects were compared by the Kruskal-Wallis test followed by the Dunn test for continuous variables and the chi-squared test for categorical variables. We analyzed the correlation of sIgE levels relative to the four material

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types using the Pearson's correlation method. Fisher's r to z transformation, a two-tailed test for independent samples, was used to assess the significance of the difference between two correlation coefficients for each of the two α -gal sIgE concentrations with two sIgE commercial kit concentrations [24]. Kappa coefficients were used to assess the diagnostic concordance of alpha-gal syndrome according to results of α -gal sIgE. We assessed and categorized the kappa value as almost perfect (0.8–1.0), substantial (0.6–0.8), moderate (0.4–0.6), fair (0.2–0.4), and poor (<0.2). Receiver operator characteristic (ROC) curve analysis was employed to identify the optimal cut-off values for α -gal sIgEs in diagnosing alpha-gal syndrome. Statistical analysis was performed using SPSS software, version 21.0 (SPSS, Inc., Chicago, IL, USA). $P \leq 0.05$ was considered statistically significant.

3 Results

3.1 Characteristics of the study subjects

As described in the methods, the subjects were divided into four different groups (Table 1). The patients with alpha-gal syndrome were significantly older than the patients in the other three groups ($P < 0.001$); however, there were no significant differences among the three non-alpha-gal groups ($P = 0.122$). Most patients, except those in the control group, were also sensitized to other common inhalants and food allergens. There were no sex differences among the four groups. We identified 23 patients who had hypersensitivity reactions after beef or pork ingestion (Table 2). Eleven patients showed urticaria within 1 h of ingestion of red meat. Among the 11 patients with an immediate hypersensitivity reaction, 7 suffered from an allergic reaction to pork only and 4 suffered from an allergic reaction to beef only. No subject showed an immediate allergic reaction to both beef and pork. Furthermore, none of the patients showed any systemic reactions such as anaphylaxis. In contrast to the immediate hypersensitivity patients, 12 patients suffered from recurrent anaphylactic reactions with skin lesions such as urticaria or angioedema after ingestion of either beef or pork, typically with a delayed onset. The range in time delay from 2 to 6 h is representative of all patients. Upon detailed questioning about their symptoms, two patients belonging to the alpha-gal syndrome group reported unique manifestations not found in the others. One patient (no. 12) reported additional anaphylaxis at the first cetuximab injection. Another patient (no. 13) described two clinical manifestations: first, itching and hives 15 min after ingestion; second, dyspnea and the spread of hives over the entire body at 3 h after ingestion. However, these reactions did not occur repeatedly.

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3.2 Total and specific IgE assay

The correlation patterns for sIgE between the beef and pork total extract and α -gal (BTG and cetuximab) are presented in Figure 1. Both BTG and cetuximab sIgE levels were highly correlated with beef sIgE levels, and comparison of the z-score showed no significant difference in the correlation coefficient between α -gal sIgE levels and the sIgE levels for beef. In addition, pork sIgE levels were significantly and highly correlated with the Sus s 1 sIgE level, whereas beef sIgE levels were not significantly correlated with Bos d 6 levels (data not shown).

Table 3 shows the results for the total IgE and sIgE assays for beef, bovine serum albumin, pork, porcine serum albumin, BTG, and cetuximab for each group. Figure 2 also shows the distributions of sIgE concentration and difference among groups. All patients with alpha-gal syndrome had significantly higher concentrations of sIgE against beef, pork, BTG, and cetuximab than that in health controls. However, these patients showed low sIgE concentrations in response to bovine and porcine serum albumin, similar to those observed in healthy controls. Individuals with beef or pork allergy and asymptomatic sensitization showed high titers of sIgE against bovine or porcine serum albumin. The sIgE against cetuximab and BTG could effectively discriminate between alpha-gal syndrome and the other three groups.

3.3 Identification of alpha-gal syndrome through sIgE

The positivity rates for each sIgE are presented in Table 3. In the asymptomatic sensitization and beef or pork immediate allergy groups, the rate of false positive results for sIgE antibodies against BTG was more than two times higher than that for sIgE against cetuximab with respect to the diagnostic criteria for alpha-gal syndrome. Specific IgE against bovine or porcine serum albumin showed significantly lower false positive rate in alpha-gal syndrome but a high false positive rate in asymptomatic sensitization. The kappa value for the diagnosis of alpha-gal syndrome according to each α -gal sIgE was higher for cetuximab (0.805; $P < 0.001$) than for BTG (0.485; $P < 0.001$). The areas under the ROC curve for sIgE for alpha-gal syndrome diagnosis were 0.964 (95% confidence interval [CI]: 0.917–1.000) for BTG and 1.000 (95% CI: 1.000–1.000) for cetuximab (Figure 3). The cut-off values that maximized the sum of sensitivity and specificity were 1.30 (sensitivity, 1.0; specificity, 0.8) for BTG and 2.30 (sensitivity, 1.0; specificity, 1.0) for cetuximab in 51 patients.

3.4 Protein and IgE binding of BTG and cetuximab

Several protein bands for BTG ranging from 35 to 180 kDa were observed on SDS-PAGE (See Supporting Information Fig 1). Two protein bands for cetuximab were observed

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on SDS-PAGE. The Mascot score of the protein band at 30 kDa analyzed by LC-coupled ESI-MA/MS was 704, corresponding with the light chain of cetuximab (gi|66361247), and the Mascot score of the protein band at 55 kDa analyzed by LC-coupled ESI-MA/MS was 715, corresponding with the heavy chain of cetuximab (gi|54305724). Consequently, the two protein bands of cetuximab on SDS-PAGE represented the putative IgG1 heavy and kappa light chains. To identify BTG and cetuximab recognized by IgE antibodies of patients with alpha-gal syndrome and to investigate the possibility of inter-individual differences, we analyzed sera from patients (BTG: 45 patients, Cetuximab: 38 patients) in immunoblots (Fig 4). Antibodies from all patients with alpha-gal syndrome and individuals with asymptomatic sensitization recognized the heavy chain of cetuximab with strong intensity in comparison with the samples from the other two groups. (Fig 4A) Except for patient no. 6, 7, and 9, the patients with immediate allergic reactions to beef or pork showed weaker bands than the samples from alpha-gal syndrome and asymptomatic sensitization. On immunoblotting of BTG (Fig 4B), most (except for patients no 7 and 8) alpha-gal syndrome samples showed several strong bands.

In the pooled serum among individuals in the asymptomatic sensitization group with positive results for both BTG and cetuximab and in individuals with alpha-gal syndrome, IgE binding with BTG was attenuated with treatment of 20 µg/mL cetuximab inhibition (Figure 5). IgE binding to cetuximab was also reduced by BTG inhibition at concentrations of 15 µg/mL and 50 µg/mL in both groups.

4 Discussion

Alpha-gal syndrome is a new type of food allergy that could be related to sensitization to the α -gal epitope [6]. α -Gal is a carbohydrate antigen that is also known to exist in red meats such as beef, pork, and lamb [5,6]. For most people, exposure to red meat results in the development of immune tolerance; however, some predisposed people show delayed-onset hypersensitivity reactions [25]. Cetuximab also contains α -gal in the murine-origin Fab region of the heavy chain, and this portion can bind to IgE specific to α -gal [26]. Although red meats and cetuximab share the α -gal epitope, the immediate allergic reaction related to cetuximab infusion is distinct from alpha-gal syndrome [25]. The time spent for digestion may be the most plausible explanation for the delayed onset of the reaction [6], whereas less time is required for the cetuximab infusion to spread to a large quantity of α -gal epitopes through the circulatory system. Nevertheless, it remains unclear as to why these allergic reactions caused by the α -gal epitope show different manifestations.

Although double-blind, placebo-controlled food challenges supervised by a physician remain the gold standard for diagnosis of food allergies, many physicians will combine a medical history with other tools [1,27] because food challenges can cause fatal adverse

reactions, such as anaphylaxis, and are quite time- and labor-intensive [28]. Specifically, the quantitative measurement of serum food sIgE levels is now routinely used in the evaluation of food allergies because of its convenience and availability [29].

Diagnosis of alpha-gal syndrome differs from that of typical food allergies in two main aspects. First, physicians and patients may find it difficult to define an allergen based on a patient's statement because the delayed reaction is far removed from a typical food allergy reaction [30]. In our study, we showed that every allergic reaction classified as alpha-gal syndrome was delayed, as previously reported [6]. Each of the patients visited the Center after they had repeatedly experienced several anaphylaxis events, and half of these patients were diagnosed with chronic idiopathic urticaria. The high incidence of chronic urticaria may be due to failure to identify the causative allergen because of the atypical manifestations of the food allergy. According to previous reports, owing to the difficulty in defining the allergen for some patients, some cases were suggested to not be alpha-gal syndrome; rather, they were regarded as idiopathic anaphylaxis [31]. The role of diagnostic methods test is crucial in this respect because of the uncertain history. Second, component-resolved diagnosis has attracted attention since the discovery of alpha-gal syndrome. After the discovery of new aspects of allergic disease, several diagnostic methods for alpha-gal syndrome were proposed in previous studies, such as an SPT, basophil activation test, and sIgE test [6,18,21]. Among them, the sIgE test has become the most useful diagnostic tool for alpha-gal syndrome, similar to other food allergies, because of the low sensitivity of SPT to commercial extracts [6,32,33]. In this study, all alpha-gal syndrome patients had a high concentration of sIgE against the total extract of beef and pork. These findings suggest that measurement of sIgE against total extract of red meats can be used to screen alpha-gal syndrome. However, only measurement of sIgE against red meat total allergens could not distinguish between subjects with typical immediate hypersensitivity to red meats and alpha-gal syndrome. Rather, addition of serum IgE against alpha-gal, bovine albumin, and beef and porcine albumin increased the accuracy of diagnosis [34]. In addition, a recent meta-analysis study demonstrated the diagnostic performance of various types of serum IgE test in patients with alpha-gal syndrome [35]. It was suggested that BTG sIgE is the most useful measurement for diagnosing alpha-gal syndrome to date [35]. Our study also corroborated the conclusions of the meta-analysis study. However, we additionally determined the importance of cetuximab sIgE for diagnosis of alpha-gal syndrome. Furthermore, we showed that measurement of Bos d 6 and Sus s 1 could discriminate alpha-gal syndrome and classic beef or pork meat allergy. In addition, all alpha-gal syndrome samples in this study had beef and pork sIgE in ImmunoCAP, compared to 89.0~92.7% in the previous meta-analysis. The difference in the sensitivity of pork or beef sIgE may have resulted from a difference in the allergic status of enrolled subjects.

Recently, a commercial sIgE measurement for α -gal was developed using BTG (ImmunoCAP o215). This product has been used in many studies investigating alpha-gal

syndrome [12,36-38]. However, no study has thus far compared the clinical usefulness of the sIgE test using other materials containing alpha-gal such as cetuximab with that using BTG.

This is the first study to investigate the usefulness of two different antigen sources containing α -gal epitopes. This sIgE test using cetuximab has many advantages for the diagnosis of alpha-gal syndrome compared to BTG sIgE. Even when using the clinical positive cut-off value (0.35 kUA/L), cetuximab sIgE showed a significantly higher inter-rater agreement than BTG sIgE. In a previous study, BTG sIgE showed the highest area under the curve among several sIgEs used for diagnosis of alpha-gal syndrome [35]. However, in the present study, according to the ROC curve, cetuximab sIgE has been suggested as a better indicator for diagnosis of alpha-gal syndrome, and the best cut-off that maximized the sum of sensitivity and specificity was determined to be 2.30 KU/L. In this study, all groups showing positive results for α -gal sIgE showed IgE reactivity to both BTG and cetuximab.

An accurate diagnosis of alpha-gal syndrome is necessary for many reasons. In addition to BTG sIgE, cetuximab sIgE can help to prevent under- and over-diagnoses of alpha-gal syndrome. Accurate diagnosis can reduce unnecessary avoidance of red meats, where the main approach to the management of food allergies depends on allergen avoidance [39]. The prevalence of alpha-gal syndrome and asymptomatic sensitization with α -gal is currently not clear, and therefore, studies are currently under way to obtain more conclusive information on alpha-gal syndrome in the general population [10,38]. Our data show that individuals with asymptomatic sensitization had various sIgEs against bovine or porcine serum albumin and alpha-gal. IgE immunoblotting and CAP data showed that all individuals with asymptomatic sensitization, and some beef or pork allergy patients, also had cetuximab sIgE in IgE immunoblotting, even though the concentrations in ImmunoCAP were undetectable or very low. The structures of cetuximab in IgE immunoblotting and ImmunoCAP are different, i.e., denatured structure and natural structure, respectively. We believe that the denatured structure of cetuximab in immunoblotting may have enhanced IgE binding, resulting in false positivity for diagnosis of alpha-gal syndrome.

We believe that quantification of cetuximab sIgE and BTG in their natural state will be helpful for researchers studying allergic reactions and sensitization to mammalian meat and lead to more accurate results. Furthermore, measurements of bovine or porcine serum albumin sIgE are expected to also be helpful for diagnosis of classic beef or pork allergy and distinguishing such allergies from alpha gal syndrome.

One interesting finding of this study is the presence of cetuximab allergy in alpha-gal syndrome. Case no. 12, a patient with alpha-gal syndrome, first visited our allergy clinic based on a referral from the oncology department. She experienced anaphylaxis at the first exposure to cetuximab treatment. While taking her detailed history, it was revealed that she had been experiencing alpha-gal syndrome for the last 3 years. Her case is unique, given that no previous study reported a history of alpha-gal syndrome in patients with cetuximab

anaphylaxis. This case emphasizes the necessity of performing a diagnostic test for α -gal sIgE using cetuximab before administration of cetuximab in patients with a suspected history of alpha-gal syndrome.

There are several limitations to our study. First, we included 23 patients showing symptoms after red meat ingestion. However, considering the current lack of information on the overall prevalence of alpha-gal syndrome and that of typical beef and pork allergy, this small number of patients was expected. Moreover, the goal of this study was to compare the usefulness of the sIgE test between cetuximab and BTG in making a diagnosis of alpha-gal syndrome, and not to estimate the prevalence of alpha-gal syndrome. Second, we did not perform any other diagnostic test such as SPT using commercial extracts. However, it is already known that the SPT and sIgE tests using commercial crude meat extracts generally yield poor or negative results in alpha-gal syndrome. Although several studies have suggested SPT using other materials to diagnose alpha-gal syndrome in several studies, we were not able to perform further SPT tests using pork kidney because of our retrospective study design. Consequently, we do not feel that this is a substantial drawback to our study. In this study, we measured BTG sIgE using a home-made kit, as the commercial alpha gal CAP (o215) was not available in Korea. Possible differences in quality between the used kit and the commercial kit may have resulted in differences in the measurement of sIgE. The final limitation is that the grouping of subjects into alpha-gal syndrome, asymptomatic sensitization, and classic pork/beef allergy groups was based on the compatibility between the clinical history and ImmunoCAP tests. However, the low prevalence of the beef and pork sIgE positivity in the general population, high cetuximab/BTG sIgE levels, and overall concordance of typical clinical symptoms strongly supported the initial diagnosis.

In conclusion, we have evaluated two different α -gal sIgE measurements. The clinical value of cetuximab and BTG sIgE levels in alpha-gal syndrome was confirmed by ImmunoCAP and inhibition immunoblot assays. Our study provides useful information for proper selection of the α -gal source material for clinical practice and research on alpha-gal syndrome. Finally, this study also confirmed the usefulness of component-resolved measurement for diagnosis of red meat-related allergic diseases.

Author contributions

D.W.S and J.S.L drafted the manuscript. D.W.S, K.H.P, Y.M.Y, J.H.L and J.W.P were involved in the clinical study of patients. D.W.S, J.S.L and K.Y.J performed the total IgE, specific IgE, and streptavidin CAP assays. D.W.S and J.S.L performed SDS-PAGE and IgE/inhibition IgE immunoblotting. D.W.S and J.W.P designed the research and reviewed the manuscript.

Conflict of interest

All authors declare no conflict of interest.

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Figure 1. Scatter plots between sIgE concentrations of the commercial kit (x-axis) and sIgE concentrations of α -gal (y-axis). The clinical cut-off values used as the diagnostic criteria for RMA based on the sIgE level to BTG and cetuximab (≥ 0.35 KU_A/L) are indicated with red lines. BTG sIgE levels were significantly and highly correlated with beef sIgE levels ($r = 0.784$; $p < 0.001$) and had a low correlation with pork sIgE levels ($r = 0.399$; $p = 0.004$). Cetuximab sIgE levels were strongly correlated with beef sIgE levels ($r = 0.669$; $p < 0.001$) and low correlated with pork sIgE levels ($r = 0.290$; $p = 0.039$).

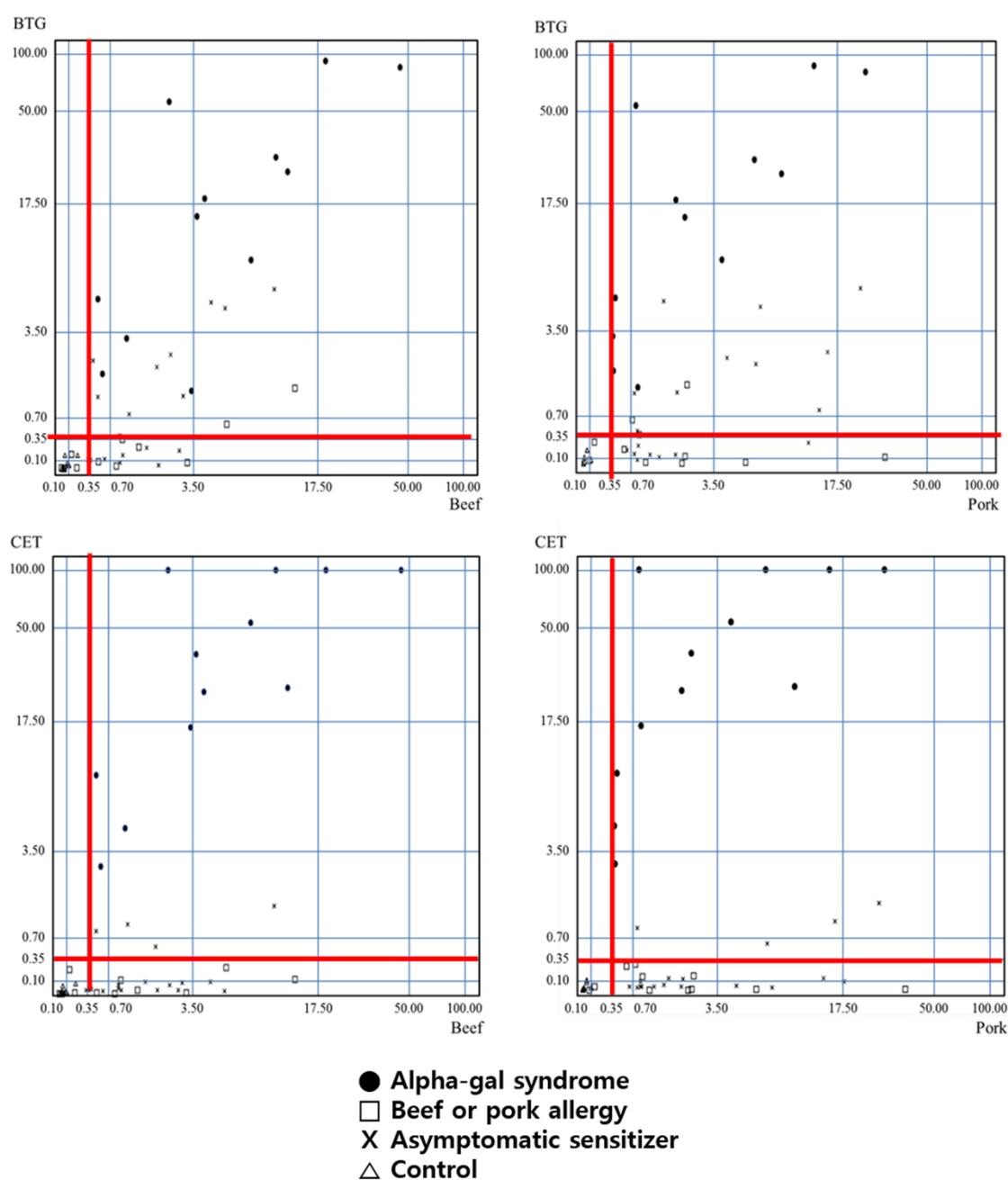


Figure 2. Concentrations of the six sIgEs (beef, pork, bovine thyroglobulin [BTG], cetuximab, Bos d 6, and Sus s 1) among the four groups ($*P < 0.05$, $**P < 0.01$ and $***P < 0.001$). Box plot showing medians and quartiles. The segment inside the rectangle shows the median and the central rectangle spans the first quartile to the third quartile. The outliers were represented by markers below the first quartile and above the third quartile.

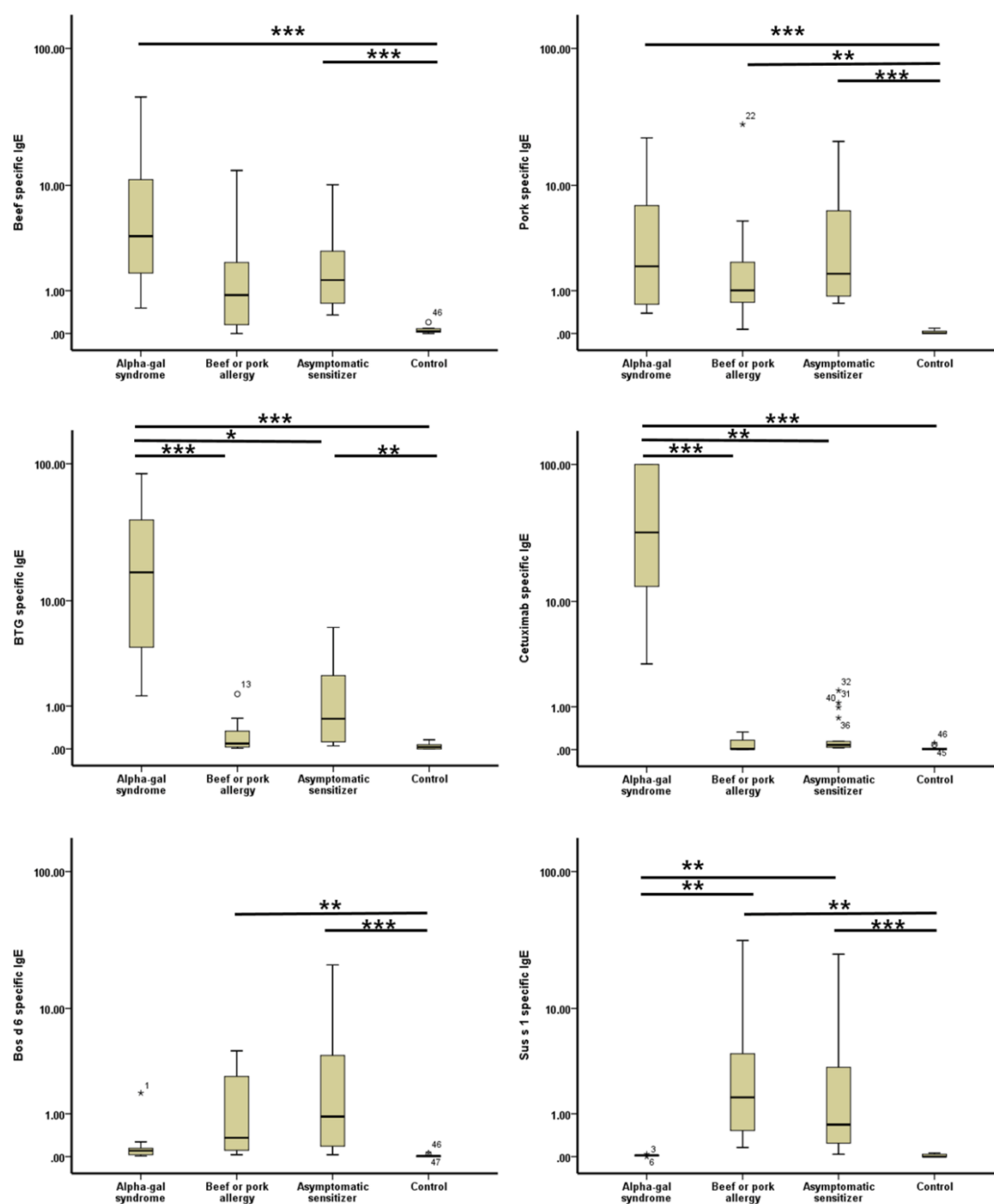


Figure 3. Receiver operating characteristic (ROC) curve of the four sIgEs used to diagnose alpha-gal syndrome. The discriminatory ability for red meat allergy was evaluated according to the area under the ROC curve for beef sIgE, pork sIgE, bovine thyroglobulin sIgE, and cetuximab sIgE for all study subjects.

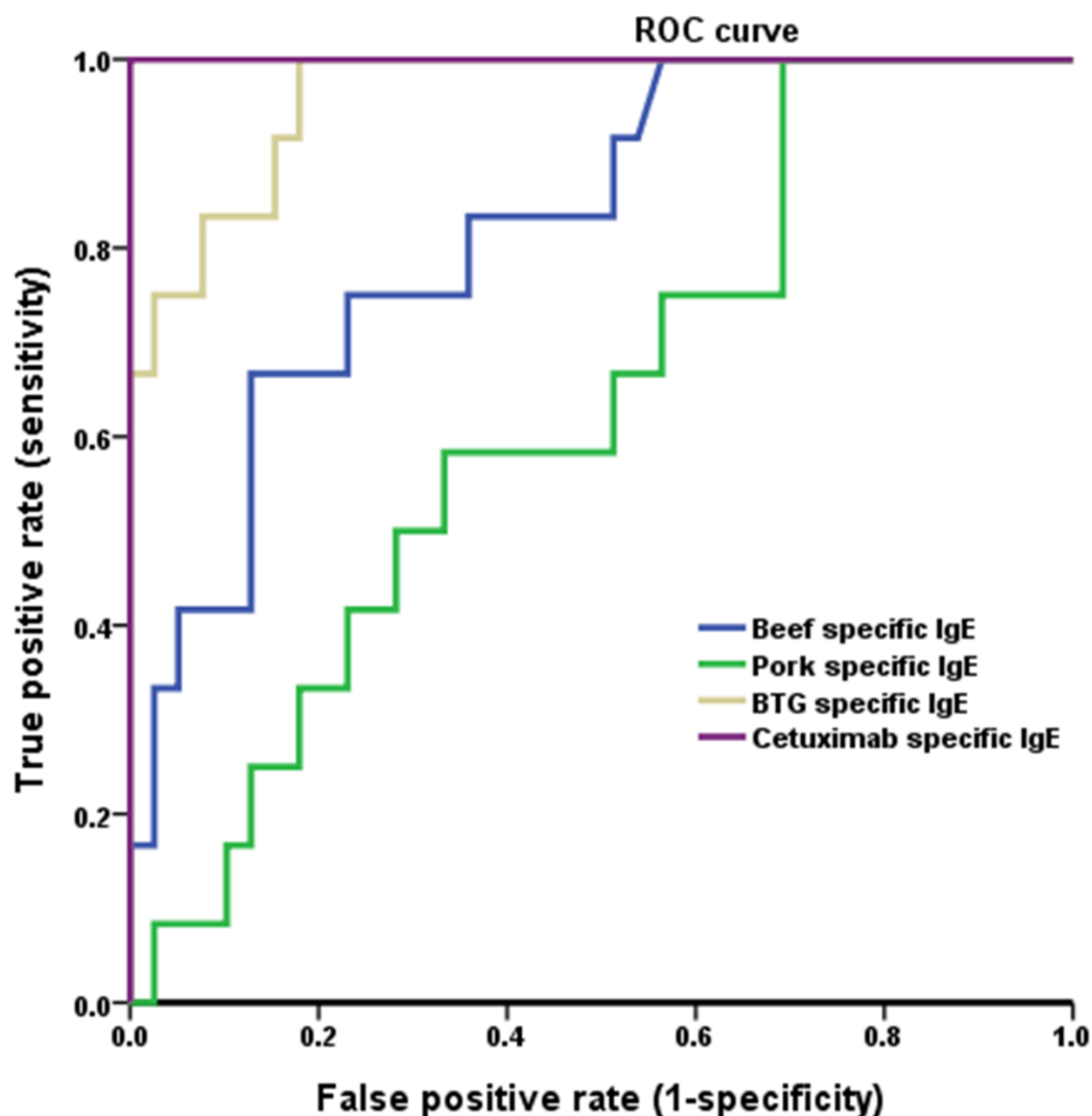


Figure 4. IgE reactivity patterns of individual patients participating in the study. Cetuximab (A) and bovine thyroglobulin (BTG) were separated under nonreducing conditions by SDS-PAGE, transferred to polyvinylidene difluoride membrane, and incubated with patient's sera.

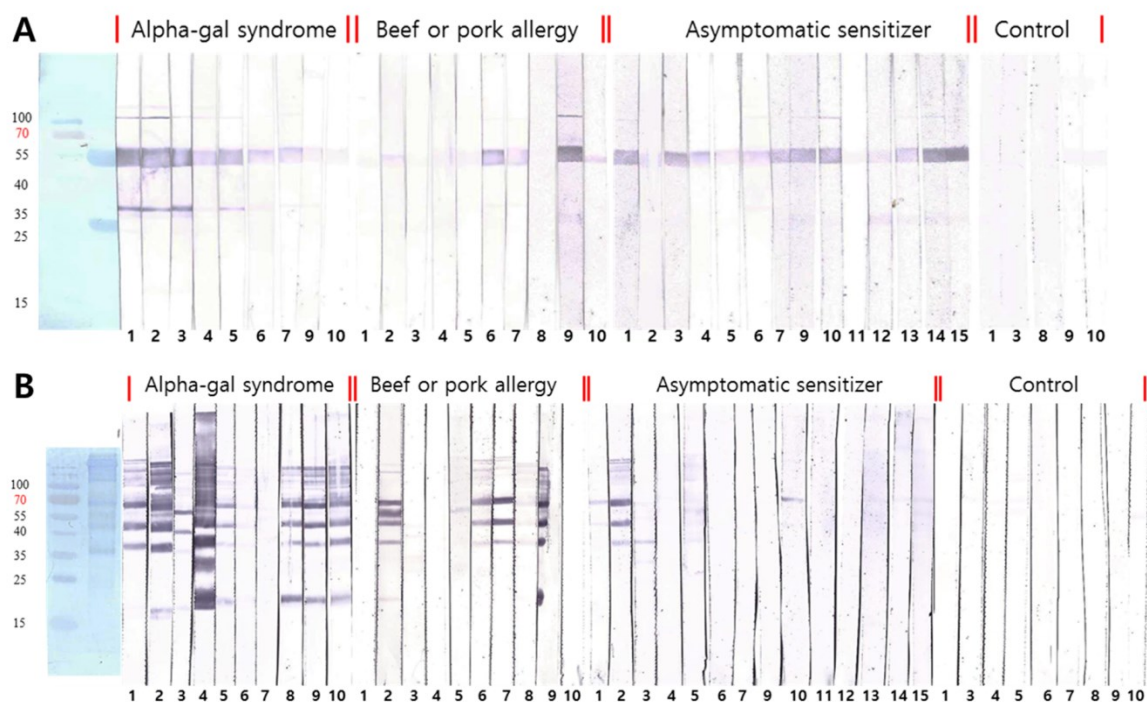


Figure 5. Inhibition immunoblot. Immunoblot inhibition analysis of binding of IgE to BTG with cetuximab in the pooled sera of the asymptomatic sensitization and alpha-gal syndrome groups revealed that BTG was attenuated by cetuximab (A). Immunoblot inhibition analysis of binding of IgE to cetuximab with BTG in the pooled sera of the asymptomatic sensitization and alpha-gal syndrome groups showed that cetuximab was also inhibited by BTG (B).

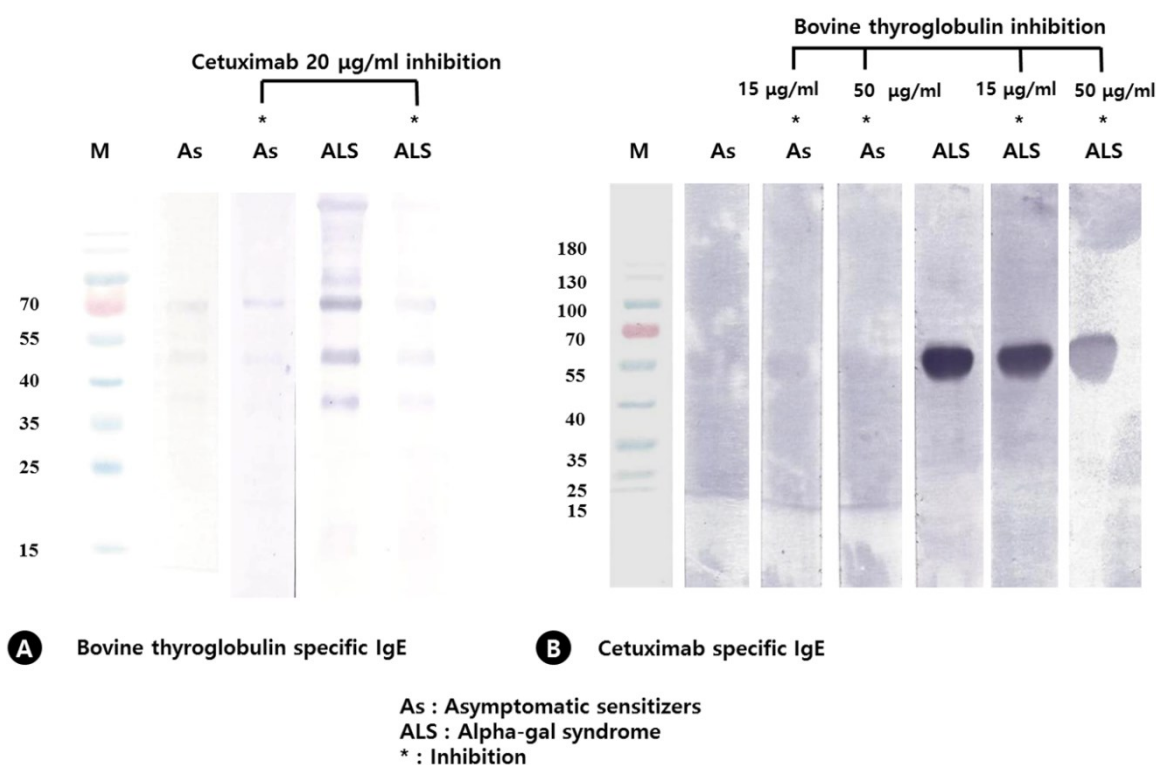


Table 1. Patient demographics.

	Group 1 Alpha-gal syndrome	Group 2 Beef or pork allergy	Group 3 Asymptomatic sensitizer	Group 4 Healthy control	<i>P</i> -value
Number of subjects	12	11	18	10	
Age, median (range)	67.0 (52.0–79.0)	18.0 (3.0–46.0)	19.5 (3.0–53.0)	28.5 (16.0–73.0)	<0.0001
Female, n (%)	5 (42%)	7 (64%)	8 (44%)	6 (60%)	0.915
Comorbidities, n (%)					
Allergic rhinitis	2 (17%)	5 (46%)	6 (33%)	0 (0%)	0.099
Asthma	2 (17%)	3 (27%)	4 (22%)	0 (0%)	0.387
Diagnosed as chronic idiopathic urticaria	5 (42%)	3 (27%)	2 (11%)	0 (0%)	0.026
Allergic conjunctivitis	0 (0%)	0 (0%)	2 (11%)	0 (0%)	0.309

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EGID	0 (0%)	1 (9%)	2 (11%)	0 (0%)	0.522
AD	0 (0%)	5 (46%)	15 (83%)	0 (0%)	<0.0001
Atopy status					
Subjects with $\geq$ one positive Immunocap result* (%)	12 (100%)	10 (91%)	18 (100%)	5 (50%)	0.001

AD, Atopic dermatitis, EGID, Eosinophilic gastrointestinal disorders.

* : The presence of other sensitizations other than beef and pork as detected through ImmunoCAP

Table 2. Characteristics of the patients reporting food allergies, including alpha-gal syndrome and typical beef and pork allergy.

No.	Age	Sex	Diagnosis	Total IgE (KU/L)	Beef sIgE (KU _A /L)	Bos d 6 sIgE (KU _A /L)	Pork sIgE (KU _A /L)	Sus s 1 sIgE (KU _A /L)	BTG sIgE (KU _A /L)	Cetuximab sIgE (KU _A /L)	Number of events*	Symptom onset after ingestion of red meat
1	27	F	Pork allergy	569.0	0.52	0.07	2.13	3.05	0.09	0.01	3	Within 15 min
2	24	F	Pork allergy	43.8	0.00	n.t	1.01	n.t	0.02	0.00	4	20–30 min
3	46	F	Pork allergy	247.0	0.03	0.03	2.02	2.29	0.01	0.00	4	Within 30 min
4	18	M	Pork allergy	3496.0	0.99	0.19	0.87	0.92	0.39	0.16	4	Within 1 h
5	31	F	Pork allergy	893.0	0.19	0.33	5.17	5.89	0.02	0.01	5	20–30 min
6	4	M	Pork	428.0	0.12	0.11	0.58	0.21	0.18	0.30	2	Within 10

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												min
7	4	F	Pork allergy	400.0	3.15	0.40	28.50	32.1	0.08	0.01	3	Within 10 min
8	3	M	Beef allergy	321.0	1.40	4.54	0.13	0.16	0.28	0.04	3	After 10 min
9	3	F	Beef allergy	110.0	0.86	2.66	0.07	n.t	0.04	0.00	2	Within 20 min
10	30	M	Beef allergy	2587.0	13.00	0.38	2.20	n.t	1.43	0.17	3	Within 30 min
11	3	F	Beef allergy	1634.0	5.48	4.33	0.73	1.07	0.64	0.33	2	Within 15 min
12	70	F	Alpha-gal syndrome	871.0	18.80	0.13	12.30	0.02	85.00	100.00	5	2–6 h
13	60	F	Alpha-gal syndrome	1140.0	7.53	0.16	3.73	0.02	8.83	55.90	3	Within 15 min, After 3 h
14	67	F	Alpha-	52.9	4.05	0.07	1.82	0.01	18.20	25.80	3	After 3 h

			gal syndrom e										
15	68	M	Alpha- gal syndrom e	355.0	0.59	0.02	0.40	0.00	1.84	3.00	4	3–4 h	
16	79	M	Alpha- gal syndrom e	470.0	10.30	n.t	5.81	n.t	29.10	100.00	4	3–4 h	
17	67	F	Alpha- gal syndrom e	2410.0	45.00	1.8	22.70	n.t	79.20	100.00	3	After 3 h	
18	60	M	Alpha- gal syndrom e	512.0	2.38	0.10	0.80	0.04	54.10	100.00	5	2–4 h	
19	58	M	Alpha- gal syndrom e	63.2	3.35	0.01	0.84	n.t	1.36	17.20	4	3–6 h	

20	67	M	Alpha-gal syndrome	197.0	11.90	0.27	8.23	n.t	24.7	27.00	10	After 2 h
21	62	M	Alpha-gal syndrome	96.0	1.09	0.02	0.39	n.t	3.18	5.07	3	2–3 h
22	52	M	Alpha-gal syndrome	510.0	3.63	0.13	2.12	0.02	14.8	39.4	3	3–4 h
23	71	F	Alpha-gal syndrome	295.0	0.51	0.04	0.43	0.02	5.42	9.82	4	After 3 h

*: Number of events before visiting the clinic.

n.t, not tested; sIgE, specific IgE.

Table 3. Total IgE and specific IgE assays.

	Group 1 Alpha-gal syndrome (n=12)	Group 2 Beef or pork allergy (n=11)	Group 3 Asymptomatic sensitizer (n=18)	Group 4 Healthy control (n=10)
Total IgE, median (range)	412.5 (52.9–2410.0)	428.0 (43.8–3496.0)	1534.0 (533.0–5000.0)	115.0 (21.3–1901.0)
Specific IgE, median (range)*				
Beef	3.84 (0.51–45.00)	0.86 (0.00–13.00)	1.39 (0.35–10.10)	0.04 (0.00–0.20)
Bovine serum albumin (Bos d 6)	0.10 (0.01–1.80)	0.36 (0.03–4.54)	0.92 (0.03–21.30)	0.10 (0.00–0.06)
Pork	1.97 (0.39–22.70)	1.01 (0.07–28.50)	1.64 (0.63–21.40)	0.01 (0.00–0.09)
Pig serum albumin (Sus s 1)	0.02 (0.00–0.04)	1.68 (0.16–32.10)	0.68 (0.04–25.50)	0.00 (0.00–0.06)
Bovine thyroglobulin	16.50 (1.36–85.00)	0.09 (0.01 –1.43)	0.64 (0.05–6.15)	0.03 (0.00–0.16)
Cetuximab	33.20 (3.00–100.00)	0.01 (0.00–0.33)	0.08 (0.03–1.60)	0.01 (0.00–0.11)

Number of positive result/ number of tests*

Beef	12/12	7/11	18/18	0/10
Bovine serum albumin (Bos d 6)	1/10	5/9	10/16	0/9
Pork	12/12	9/11	18/18	0/10
Pig serum albumin (Sus s 1)	0/7	5/7	10/16	0/9
Bovine thyroglobulin	12/12	3/11	10/18	0/10
Cetuximab	12/12	0/11	4/18	0/10

* : Beef, pork, bovine thyroglobulin, and cetuximab sIgE assays were performed for all study populations; Bos d 6 and Sus s 1 were only performed for some of the study populations.